

## Formation, isolation and characterization of a prolyl tripeptidyl peptidase of *Porphyromonas gingivalis*

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### Summary

Formation, cellular locations, isolation and enzymatic properties of PTP of *Porphyromonas gingivalis*, an anaerobic periodontal pathogen were investigated.

Almost all activities of this enzyme were detected in the crude extract of the cell, but the other bacterial fractions such as culture fluids, envelopes and vesicles were not found to contain PTP.

PTP was purified from the crude extract prepared by sonication and centrifugation through five steps including concentration, ion exchange chromatography, gel filtration, hydrophobic interaction chromatography and isoelectric focusing to homogeneity.

The enzyme was a serine enzyme since it was inhibited strongly by Pefabloc SC, diisopropyl fluorophosphate and 3,4-dichloroisocoumarin. It hydrolyzed H-Ala-Ala-Pro-pNA and H-Ala-Phe-Pro-pNA. The molecular mass was determined as 45 kDa and isoelectric point was 5.7.

Optimum pH was moderately broad, and maximum activity was observed in the range of pH 7.0 to 9.0.

The residual activity after heating at 50°C for 5 min was 29%, but heating at 60°C resulted in complete loss of the activity.

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Abbreviations: dipeptidyl peptidase, DPP; tripeptidyl peptidase, TPP; prolyl tripeptidyl peptidase, PTP; p-nitroanilide, pNA; arginine gingipain, RGP; lysine gingipain, KGP; 50 mM Tris-HCl buffer (pH 8.2), Tris-HCl buffer

## Introduction

Gram-negative, black-pigmented obligatory anaerobes including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* have been considered putative etiological agents of human periodontitis, of which *P. gingivalis* is the most potent pathogen of this disease, based on ecological properties in the oral cavity and etiological characteristics including production of proteolytic enzymes<sup>1-6)</sup> and endotoxin<sup>7-10)</sup>. In addition, DNAs of the periodontopathogenic bacteria were detected from oral samples of patients with Buerger disease (obstructive thromboangitis) and these patients had moderate to severe periodontitis<sup>11)</sup>. These findings suggest that the periodontopathogens are implicated as causative agents of systemic disease as well as of oral disease.

As pathogenic factors of *P. gingivalis* (formerly *Bacteroides gingivalis*), proteinases (RGP and KGP), once designated "trypsin-like enzymes"<sup>12)</sup> have been most intensively studied<sup>13-17)</sup>. *P. gingivalis*, is an asaccharolytic organism, utilizes peptides but not free amino acids as its energy source<sup>18-21)</sup>. Proteins surrounding the organisms are degraded by these proteinases to fragments which must be further hydrolyzed into smaller peptides by peptidases to provide adequate nutrients and energy sources. In this sense, peptidases of *P. gingivalis* are significant enzymes.

Previously we reported the isolation and characterization of DPP degraded H-Lys-Ala-pNA with weak activity against H-Ala-Ala-pNA and H-Val-Ala-pNA of *P. gingivalis*<sup>22)</sup>. Suido et al. pointed out that *P. gingivalis* actively elaborated many kinds of DPPs, but only poor TPPs were found<sup>23)</sup>. However, PTP activity was confirmed in the soluble fraction of the cell of *P. gingivalis* ATCC 33277, thus we will describe formation, isolation and properties of PTP of this species in this report.

## Materials and Methods

### **Bacterial strains and cultivation methods**

*Porphyromonas gingivalis* ATCC 33277 was mainly used through the studies. *P. gingivalis* 381, W50 and W83 were also employed for additional studies. These strains were maintained on blood agar plates and grown in a liquid medium consisting of trypticase peptone (17 g/l), yeast extract (3 g/l), NaCl (5 g/l), K<sub>2</sub>HPO<sub>4</sub> (2.5 g/l), hemin (5 mg/l) and menadione (0.5 mg/l)<sup>24)</sup>. Cultivation was carried out anaerobically in a glove box filled with a mixture of gasses containing N<sub>2</sub>: H<sub>2</sub>: CO<sub>2</sub>=85: 10: 5 at 37°C for 3 days. Bacterial growth was monitored by optical density at 600 nm (OD<sub>600</sub>).

### **Assays**

Protein concentration was determined by absorbance at 280 nm, using bovine serum albumin as a standard protein ( $A_{280}$  of 1mg/ml bovine serum albumin solution=0.624).

The substrates for DPP and PTP, *p*-nitroanilide derivatives of amino acids and peptides, were obtained from Bachem, AG, Bubendorf, Switzerland, and Peptide Institute, Inc. Osaka, Japan. The peptidase activities were determined photometrically<sup>25,26)</sup>. One unit of the enzyme activity was defined as the liberation of 1 μmol of *p*-nitroaniline per min as described earlier<sup>14,16)</sup>. Reaction mixtures containing 50 μl of enzyme source, 700 μl of 1 mM substrates in 50 mM Tris-maleate buffer (pH 7.5) and 150 μl of 50 mM Tris-maleate buffer (pH 7.5) were incubated at 37°C for 30 min. To stop the reaction, 100 μl of 7.5 M acetic acid was added, and released *p*-nitroaniline was assayed by

absorbance at 410 nm. H-Ala-Ala-Pro-pNA was used routinely to assay PTP activity, if not otherwise stated.

RGP and KGP were determined using Bz-Arg-pNA and Tos-Gly-Pro-Lys-pNA, respectively<sup>13,25</sup>.

Non-specific proteolytic activity was measured using azocoll as a substrate. The reaction mixtures containing 4 mg of the substrate, 100  $\mu$ l of enzyme source and 900  $\mu$ l of 50 mM Tris-maleate buffer (pH 7.5) were incubated at 37°C for 30 min. After incubation, reaction mixtures were cooled immediately in an ice-water bath to stop the reactions, followed by centrifugation at 15,000 xg at 4 °C for 3 min. The absorbance of the supernatants at 520 nm ( $A_{520}$ ) was measured, and the activity was defined as the increase of the  $A_{520}$  by 1.0 per min. Similarly, hydrolysis of remazol brilliant blue hide powder (RBB-hide powder) was evaluated by increase at  $A_{595}$ .

### ***Preparation of bacterial fractions***

All procedures described below were performed at 4 °C.

The cells harvested from 2,000 ml culture by centrifugation at 10,000 xg for 15 min [11.7 g (wet weight)] were suspended in Tris-HCl buffer and subjected to sonication at 150 W for 15 min. After centrifugation of the sonicate at 6,000 xg for 15 min to remove cell debris and unbroken cells, the supernatant was centrifuged at 100,000 xg for 60 min. The supernatant and precipitate (2.0 g) were designated the crude extract and the envelope, respectively. Meanwhile ammonium sulfate was added to the supernatant of the whole culture at 40% saturation of this drug to prepare the vesicle fraction, and stirred for 5 h followed by centrifugation at 10,000 xg for 20 min. The precipitate (220 mg) was suspended in Tris-HCl buffer and dialyzed against the same buffer and referred to as the vesicle<sup>27</sup>.

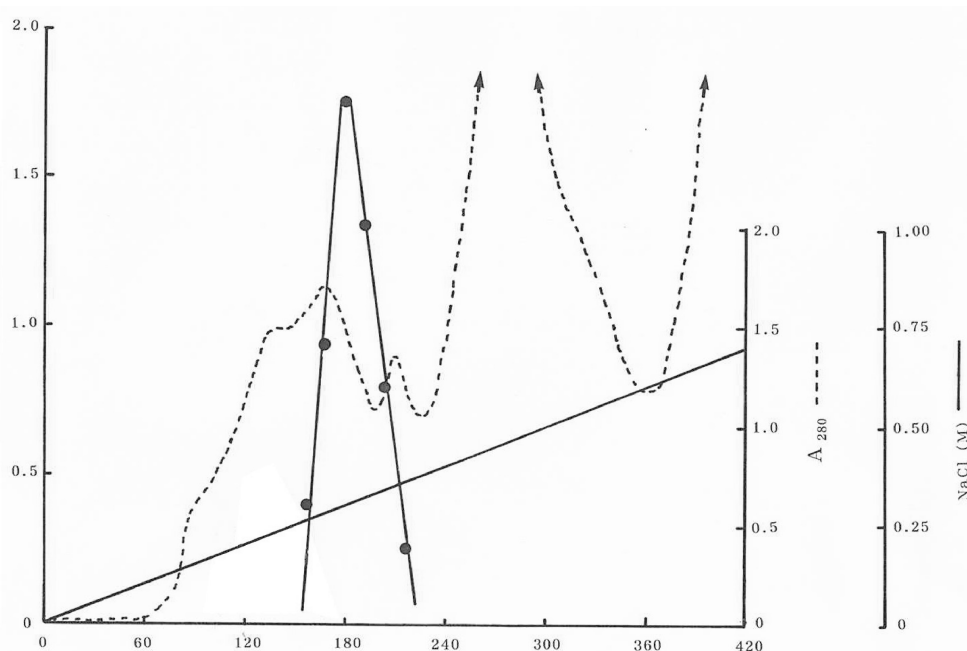
The concentration of ammonium sulfate of the supernatant was brought to 75% saturation and stirred for 8 h. The mixture was centrifuged at 10,000 xg for 20 min and dissolved in Tris-HCl buffer and dialyzed against the same buffer, and designated concentrated culture supernatant.

### ***Extraction of TPP from cells***

Cells were suspended in Tris-HCl buffer at a concentration of 50 mg/ml. Several reagents were added separately to the aliquots of this suspension and the mixtures were shaken gently at room temperature for 1 h followed by centrifugation at 30,000 xg for 30 min. The ratios of the activities in each supernatant to that of sonicate were compared.

### ***Purification***

To prepare the starting material of purification of PTP, ammonium sulfate was added to crude extract of strain ATCC 33277 at 75% saturation and stirred for 8 h, followed by correction of precipitate by centrifugation at 10,000 xg for 15 min and dialysis against Tris-HCl buffer as described above for the preparation of concentrated culture supernatant. The concentrated material was applied to a column of Q-Sepharose previously equilibrated with Tris-HCl buffer. After the column was rinsed thoroughly, proteins adsorbed to the column were developed with linear gradient of NaCl in Tris-HCl buffer from 0 to 500 mM (Fig. 1). PTP active fractions eluted with around 300 mM NaCl were combined, concentrated in vacuo and dialyzed against Tris-HCl buffer containing 150 mM NaCl. The active fractions were concentrated in vacuo, dialyzed against Tris-HCl buffer containing 150 mM NaCl, subjected to gel filtration on Sephacryl S-300 equilibrated with the same



**Fig. 1** : Q-Sepharose chromatography.  
 Symbols: ●—● ; PTP activity, - - - - ;  $A_{280}$ , — ; NaCl.

buffer saline. The active fractions from gel filtration procedure were collected and dialyzed against Tris-HCl buffer containing 700 mM ammonium sulfate and applied to hydrophobic interaction chromatography on a Phenyl Sepharose CL-4B column equilibrated with Tris-HCl buffer containing 700 mM ammonium sulfate. When the column was eluted with a descending gradient of ammonium sulfate from 700 mM to 0 mM, PTP activity was detected in the fractions eluted with about 50 mM ammonium sulfate. The active fractions were dialyzed against 150 mM glycine solution and further purified by isoelectric focusing at 300 V for 24 h using ampholine generating a pH gradient from 3 to 10 in the column. In this procedure, PTP was found to be focused at pH 5.7 zone (Fig. 2). This fraction was dialyzed against Tris-HCl buffer and referred to as the purified PTP.

### ***Sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE)***

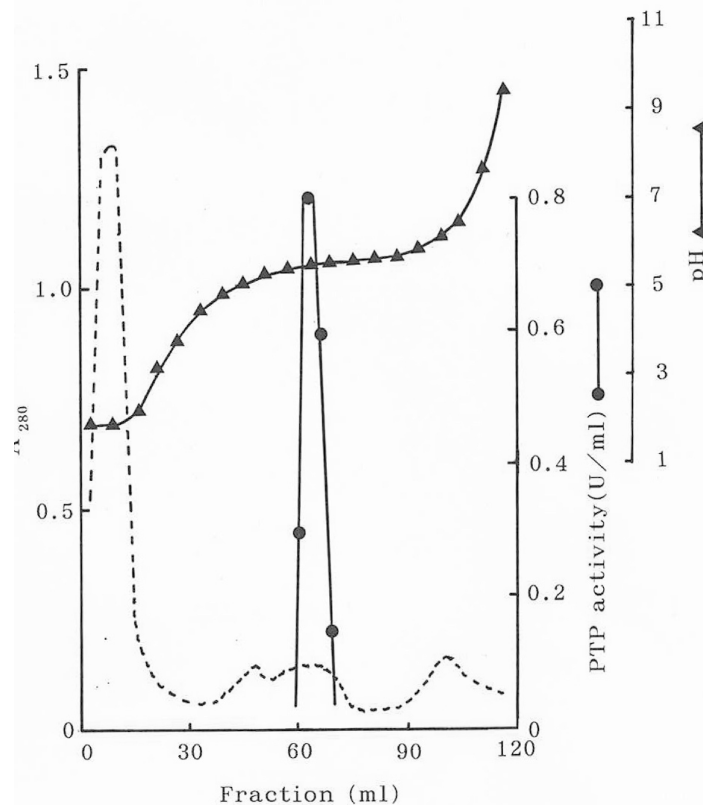
SDS-PAGE was employed to monitor enzyme purification and determination of molecular mass<sup>28</sup>). Concentration of acrylamide was 12.5% and the gels were stained with Coomassie brilliant blue R-250. The marker proteins for the reference of molecular mass estimation were phosphorylase *b* (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and *a*-lactalbumin (14.4 kDa), which were purchased from GE Healthcare, Buckinghamshire, UK.

### ***Effects of group specific reagents on PTP***

After incubation of mixtures of various group specific reagents and purified samples at 37°C for 15 min, the residual enzyme activities were compared with the control without reagent treatment.

### ***Optimum pH for the enzyme activity***

For evaluation of the influence differences in pH values of the incubation mixtures of the en-



**Fig. 2 :** Isoelectric focusing.  
 Symbols: ●—● ; PTP activity, ▲—▲ ; pH, .....;  $A_{280}$ .

zyme activity, the following buffers were introduced into the reaction mixtures to a final concentration at 100 mM: acetate buffer (pH 4 to 6), Tris–maleate buffer (pH 6.5 to 7.5), Tris–HCl buffer (pH 8 to 9) and carbonate–bicarbonate buffer (pH 9.5 to 10).

## Results

### *Time course of growth and PTP production*

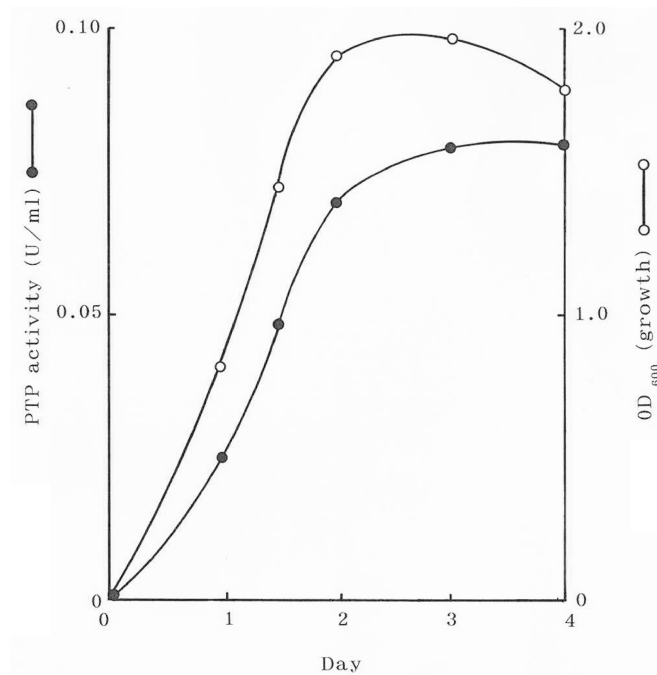
As illustrated in Fig. 3, daily progress of cell growth and PTP production were examined. Both growth and production continued vigorously till Day 2 of the cultivation. After that PTP production reached a plateau. However, growth ceased and tended toward decrease by lysis of cells.

### *Extraction of PTP from cells*

From the evaluation of cellular locations of PTP, the enzyme was found to remain in the cytoplasm. Efforts were made to search for effective means of extraction of the enzyme using several reagents. The most adequate extraction was obtained by two detergents, 3-[3-cholamidopropyl]-dimethylammonio]-propanesulfonate (CHAPS) and Triton X-100. However, mechanical disruption by sonication resulted in the highest yield of PTP (Table 1).

### *Cellular locations of PTP*

Nearly all PTP activity was detected in the crude extract prepared by sonication and centrifugation. While the base of PTP activity in the crude extract was 100, levels in the envelope, vesicle



**Fig. 3** : Time course of production of PTP and growth.  
 Symbols: ●—● ; PTP activity, ○—○ ; growth.

**Table 1** : Extraction of PTP from cells.

Treatment	PTP recovery (%)
Tris-HCl buffer	3.1
NaCl (150mM)	10.8
EDTA (10mM)	5.5
Mercaptoethanol (15mM)	5.0
CHAPS (0.5%)	42.9
Triton X-100 (0.5%)	37.7
Sonication	100.0

and culture fluid were only 4.1, 0.3 and 0.3, respectively.

### **Comparison of PTP production by 4 strains of *P. gingivalis***

PTP activities in the crude extracts prepared by sonication and centrifugation from 2.5 g of *P. gingivalis* strains of ATCC 33277, 381, W50 and W83 were compared. The extracts of all the strains were shown to elaborate this enzyme. The total units of PTP in each extract were 27.3 U, 10.8 U, 23.0 U and 15.7 U, respectively.

### **Degradation of *p*-nitroanilide derivatives of amino acids and peptides**

Among the tested synthetic and dye-conjugated protein substrates for peptidase and proteinase, respectively, the crude extract degraded H-Ala-Ala-Pro-pNA, H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, H-Arg-pNA, H-Lys-pNA, H-Lys-Ala-pNA, Bz-Arg-pNA, Bz-Lys-pNA, Tos-Gly-Pro-Lys-pNA, Azocoll and RBB-hide powder. However, H-Leu-pNA, H-Ala-Ala-Ala-pNA, Met-Ala-Ala-Pro-Val-pNA or Glt-Ala-Ala-Pro-Leu-pNA were not hydrolyzed.

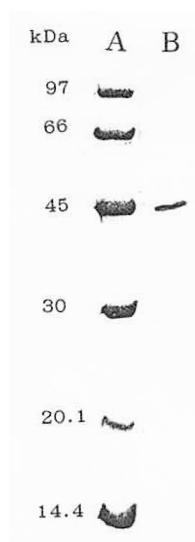
### **Purification and purity of PTP**

PTP was purified from the crude extract of cells through six steps. The enzyme was purified

**Table 2** : Purification of PTP of *P. gingivalis* ATCC 33277.

Step	protein (mg)	total activity (U)	sp. act * (U/mg)	purification (fold)	recovery (%)
Crude extract	184.8	19,888	0.009	1.0	100.0
Ammonium sulfate	120.1	8,928	0.013	1.4	65.0
Q-Sepharose	33.8	609	0.056	6.2	18.3
Sephacryl S-300	13.1	12.5	1.050	117.0	7.1
Phenyl Sepharose	8.8	3.9	2.256	251.0	4.7
Isoelectric focusing	2.4	0.65	3.090	410.0	1.3

\*: specific activity (U/mg protein)



**Fig. 4** : SDS-PAGE of purified PTP.  
Lane A, marker proteins; Lane B, purified PTP.

410 fold with a recovery of 1.3%. The purification is summarized in Table 2. SDS-PAGE analysis of the purified sample by isoelectric focusing revealed a single stained band. The molecular mass was calculated as 45 kDa (Fig. 4).

### ***Inhibition by various reagents of PTP***

Effects of group specific reagents on PTP are summarized in Table 3. Significant inhibition of PTP was observed by serine enzyme inhibitors such as Pefabloc SC<sup>2)</sup>, diisopropyl fluorophosphate and 3,4-dichloroisocoumarin. No effect was noticed by other chemicals.

### ***Substrate specificity and kinetic constants***

Among tested *p*-nitroanilide derivatives of amino acids and peptides, the purified enzyme only actively hydrolyzed Ala-Ala-Pro-pNA and Ala-Phe-Pro-pNA. Kinetic constants for two substrates are presented in Table 4. H-Ala-Ala-Ala-pNA, H-Ala-Ala-Phe-pNA, H-Gly-Phe-pNA, H-Lys-Ala-pNA, H-Gly-Pro-pNA, Bz-Arg-pNA, Tos-Gly-Pro-Lys-pNA, Suc-Ala-Ala-Pro-pNA and Suc-Ala-Ala-Ala-pNA were not degraded. Neither azocoll nor remazol brilliant blue hide powder was hydrolyzed.

### ***Thermostability of the enzyme***

When the enzyme was heated at 50°C for 5 min, the residual activity was 29% for the unheated



**Table 3** : Effects of group specific reagents on PTP.

Reagent	Concentration	Activity (%)
Control	—	100
Leupeptin	2mM	100
Antipain	10mM	100
Bestatin	2mM	106
E64 <sup>1)</sup>	2mM	114
Pefabloc SC <sup>2)</sup>	2mM	5
Diisopropyl fluorophosphate	10mM	13
3,4-Dichloroisocoumarin	2mM	6
EDTA	10mM	97
1,10-Phenanthroline	2mM	93
Mercaptoethanol	2mM	98
SDS	0.5%	83

1): L-*trans*-epoxy-succinyl leucylamido-(4-guanidino)butane

2): 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride

**Table 4** : Kinetic constants.

Substrate	Activity (%)	Km (mM)	Vmax (U/mg/min)	Vmax/Km
H-Ala-Ala-Pro-pNA	100	0.16	1.14	7.13
H-Ala-Phe-Pro-pNA	83	0.14	0.59	4.21

sample. Treatment at 60°C for 5 min resulted in complete inactivation.

### Optimum pH

Optimum pH for the enzyme activity was seen from pH 7.0 to 9.0. However, the activity decreased rapidly in the ranges of lower than pH 6.5 and higher than 9.0. The similar results were observed using H-Ala-Phe-Pro-pNA as substrate in substitution for H-Ala-Ala-pNA.

### Discussion

We isolated PTP from the crude extract of *P. gingivalis* by chromatography, gel filtration and isoelectric focusing. Among the purification steps, gel filtration on Sephacryl S-300 was rather effective, and the specific activity rose about 19 fold by this means. Since the enzyme was inhibited strongly by Pefabloc SC, diisopropyl fluorophosphate and 3,4-dichloroisocoumarin, PTP is a serine enzyme, as are other peptidases of this species<sup>29-32)</sup>.

As shown in PTP of *P. gingivalis*, bacterial peptidases are generally, not sensitive to inhibition by metal chelators. Exceptionally, PTP of *S. anginosus*, a metalloenzyme, was inhibited by EDTA, EGTA and 1,10-phenanthroline<sup>26)</sup>.

PTP investigated in the present report is an exopeptidase since peptides with a blocked amino group are not hydrolyzed, and it requires proline residue in the P1 position.

PTP of *Prevotella nigrescens* which is a quite close species of *P. intermedia*<sup>33)</sup> was isolated and characterized<sup>34)</sup>. It also was a serine enzyme and degraded H-Ala-Ala-Pro-pNA and H-Ala-Phe-Pro-pNA like PTP of *P. gingivalis*. However, differences are apparent in the molecular weights and pH-activity relationship<sup>34)</sup>.

PTP of *P. gingivalis* was already purified from the cell extract using Triton X-100 and precisely characterized<sup>32)</sup>. This enzyme had two molecular masses, 81.8 kDa and 75.8 kDa. The authors cor-



roborated that the smaller molecule was possibly derived from the larger one by proteolytic truncation of 6 kDa amino-terminal peptide. However, we found only one molecule, and its molecular mass was estimated as 45 kDa by SDS-PAGE. Even though the reason for this discrepancy (in the molecular masses) is still obscure, possible explanations include the different methods of preparation of the starting material for purification: chemical extraction by detergent and mechanical extraction by sonication. Furthermore, different strains of the source of the starting material for purification may also be a likely reason for this discrepancy, since three strains of *P. gingivalis* (HG66, W50 and ATCC 33277) were used in the cellular location tests of PTP, but it can not be specified which strain was used for the purification of PTP from the description of this report. In this report, optimum pH for the activity determined using H-Ala-Phe-Pro-pNA was found in the range of 6.0 to 8.0. This value nearly coincided with our results.

We confirmed also that PTP was extractable by detergent treatment including Triton X-100 (Table 1) but recovery rate appeared to be significantly lower than with treatment by sonication.

PTP degrades the fragments of proteins occurred by the endogenous proteinases and generates the smaller peptides in collaboration with other peptidases. These peptides may provide energy source for *P. gingivalis* which is asaccharolytic and incapable of transporting free amino acids<sup>35</sup>.

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抄録： *Porphyromonas gingivalis* のプロリルトリペプチジルペプチダーゼの産生，単離および性状

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偏性嫌気性菌周病原菌 *Porphyromonas gingivalis* のプロリルトリペプチジルペプチダーゼ (PTP) の産生，単離と酵素性状について検討した。

PTP活性のほとんどは，細胞の粗抽出液に検出され，培養上清，エンベロープやベジクルには極僅かしか分布していなかった。菌体の超音波処理と100,000xg遠心で得た粗抽出液を出発材料にして，PTPを硫酸アンモニウムによる塩析濃縮，イオン交換クロマトグラフィー，ゲル濾過，疎水性クロマトグラフィー，等電点電気泳動にて精製したところ，PTPはPefabloc Sc, diisopropyl fluorophosphate, 3,4-dichloroisocoumarinで強く阻害されたので，セリン酵素に分類される。

分子量は45 kDaで等電点は5.7であった。精製標品はH-Ala-Ala-Pro-pNAとH-Ala-Phe-Pro-pNAに活性を示し，それぞれのVmax (U/mg/min)/Km (mM) は7.13および4.21であった。反応の至適pHは7.0~9.0に見られた。熱安定性試験では，50℃，5分，加熱後の残存活性は29%であったが，60℃，5分では，完全失活することが分かった。

本研究で扱った *P. gingivalis* のPTPは，セリン酵素に分類されるプロリン特異的エキソペプチダーゼである。この酵素は，菌の栄養素の摂取上有用と考えられ，その生存に寄与し，延いては，病原性の一因子となる可能性がある。